

B5

(2)

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
18 December 2003 (18.12.2003)

PCT

(10) International Publication Number  
WO 03/104445 A1 = 21290

- (51) International Patent Classification<sup>7</sup>: C12N 9/02, C12P 7/60, 1/04, C12R 1/02 (74) Agent: PANES, Nigel; 124 Grenzacherstrasse, CH-4070 Basle (CH).
- (21) International Application Number: PCT/EP03/05676 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 30 May 2003 (30.05.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 02012582.9 6 June 2002 (06.06.2002) EP (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): ROCHE VITAMINS AG [CH/CH]; 124 Grenzacherstrasse, CH-4070 Basle (CH).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): HOSHINO, Tatsuo [JP/JP]; 2-18-14, Fueta, Kamakura-shi, Kanagawa-ken 248-0027 (JP). MIYAZAKI, Taro [JP/JP]; Kameino 2-14-8, Fujisawa-shi, Kanagawa-ken 252-0813 (JP). SUGISAWA, Teruhide [JP/JP]; Kohnandai 8-26-10, Kohnan-Ku, Yokohama-shi, Kanagawa-Ken 234-0054 (JP).

## Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ALDEHYDE DEHYDROGENASE

(57) Abstract: The present invention concerns a novel aldehyde dehydrogenase having the following physico-chemical properties: a molecular weight of  $190,000 \pm 15,000$  Da which comprises a subunit structure of two  $\alpha$  subunits and one  $\beta$  subunit, or a molecular weight of  $250,000 \pm 20,000$  Da which comprises a subunit structure of two  $\alpha$  subunits and two  $\beta$  subunits, in which the  $\alpha$  subunit has a molecular weight of  $75,000 \pm 3,000$  Da and the  $\beta$  subunit has a molecular weight of  $55,000 \pm 2,000$  Da; dehydrogenase activity on L-sorbose, D-glucosone, D-glucose and D-xylose; utilizes as cofactor pyrroloquinoline quinone and heme c; has an optimum pH of from about 6.5 to about 8.0 for the production of vitamin C and an optimum pH of about 9.0 for the production of 2-keto-L-gulonic acid from L-sorbose; and is inhibited by  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , monoiodoacetate, and sodium azide.

WO 03/104445 A1

Aldehyde dehydrogenase

The present invention concerns a novel enzyme, namely aldehyde dehydrogenase (hereinafter referred to as SNDH III), which is responsible for both of the conversions, from L-sorbose to L-ascorbic acid (hereinafter referred to as vitamin C) at neutral pH, and from L-sorbose to 2-keto-L-gulonic acid (hereinafter referred to as 2-KGA) at alkaline pH. The present invention also provides a process for producing said enzyme and a process for producing vitamin C and/or 2-KGA directly from aldoses such as L-sorbose utilizing said enzyme.

Vitamin C is one of very important and indispensable nutrient factor for human beings. The metabolic pathways to produce vitamin C have been widely studied in various organisms. However, there is no report about purified enzymes relating to the direct conversion of L-sorbose to vitamin C. Therefore, the enzyme of the present invention is very useful for a novel vitamin C production process substitutive for the current process such as the Reichstein method (Helvetica Chimica Acta 17:311 (1934)).

The present invention provides a purified aldehyde dehydrogenase having the following physico-chemical properties:

- a) Molecular weight of  $190,000 \pm 15,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and one  $\beta$  subunit) or molecular weight of  $250,000 \pm 20,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and two  $\beta$  subunits), wherein the  $\alpha$  subunit has a molecular weight of  $75,000 \pm 3,000$  Da and the  $\beta$  subunit has a molecular weight of  $55,000 \pm 2,000$  Da;
- b) Substrate specificity: active on aldehyde compounds,
- c) Cofactors: pyrroloquinoline quinone (PQQ) and heme c,
- d) Optimum pH: from about 6.5 to about 8.0 (for the production of vitamin C from L-sorbose) or about 9.0 (for the production of 2-keto-L-gulonic acid from L-sorbose),
- e) Inhibitors:  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , monoiodoacetate and sodium azide.

In one embodiment, the present invention is related to an aldehyde dehydrogenase with a molecular weight of  $190,000 \pm 15,000$  Da having the physico-chemical properties as described above.

In a further embodiment, the present invention is related to an aldehyde dehydrogenase with a molecular weight of  $250,000 \pm 20,000$  Da having the physico-chemical properties as described above.

The source of the SNDH III of the present invention is not critical. Thus, SNDH  
5 III of the present invention can be produced, for example, by isolation from a *Gluconobacter* or another microorganism capable of producing the aldehyde dehydrogenase having the above properties or it can be produced recombinantly or by chemical synthesis.

Another object of the present invention provides a process for producing SNDH III  
10 described above, comprising cultivating a microorganism belonging to the genus *Gluconobacter*, which is capable of producing the aldehyde dehydrogenase having the above mentioned properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism, and isolating the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism.

15 In one aspect of the present invention, the process for producing SNDH III as described above is carried out by cultivating a microorganism belonging to the genus *Gluconobacter*, which is capable of producing the aldehyde dehydrogenase having the above mentioned properties, wherein the reaction is carried out at a pH of from about 4.5 to about 9.0 and at a temperature of from about 20 to about 50°C. The SNDH III  
20 thus produced is useful for both the production of vitamin C and 2-KGA.

Further object of the present invention provides a process for producing a carboxylic acid and/or its lactone from its corresponding aldose, comprising contacting the aldehyde with the purified SNDH III having the above mentioned properties, or cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* which  
25 is capable of producing the aldehyde dehydrogenase having the above mentioned properties in the presence of an electron acceptor.

The term "aldose" means an aldehyde with a hydroxyl group on every carbon atom except the carbonyl carbon atom.

The aldoses as used herein include but are not limited to L-sorbose, D-glucosone, D-glucose, and D-xylose.  
30

A preferred lactone is vitamin C, a preferred carboxylic acid is 2-KGA and a preferred aldose is L-sorbose.

In one embodiment, the process for producing a carboxylic acid and/or its lactone from its corresponding aldose comprises contacting the aldehyde with the purified SNDH III having the above mentioned properties or with a cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* as defined above, wherein  
5 the molecular weight of said SNDH III is  $190,000 \pm 15,000$  Da.

In one embodiment, the process for producing a carboxylic acid and/or its lactone from its corresponding aldose comprises contacting the aldehyde with the purified SNDH III having the above mentioned properties or with a cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* as defined above, wherein  
10 the molecular weight of said SNDH III is  $250,000 \pm 20,000$  Da.

In one aspect, the present invention is directed to a process for producing a carboxylic acid and/or its lactone from its corresponding aldose comprising contacting the aldehyde with the purified SNDH III having the above mentioned properties or with a cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter*,  
15 which is capable of producing the aldehyde dehydrogenase having the above mentioned properties, wherein the reaction is carried out at a pH of from about 4.5 to about 9.0 and at a temperature of from about 20 to about 50°C. In case of the production of vitamin C, the reaction is carried out preferably at a pH of from about 6.5 to about 8.0. In case of the production of 2-KGA, the reaction is carried out preferably at a pH of about 9.0.

20 The invention also features the use of the purified aldehyde dehydrogenase having the above mentioned properties in the process for the production of a carboxylic acid and/or its lactone from its corresponding aldose which comprises contacting the aldehyde with said purified aldehyde dehydrogenase or cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* which is capable of producing said  
25 aldehyde dehydrogenase in the presence of an electron acceptor.

The physico-chemical properties of the purified sample of SNDH III prepared according to the Examples mentioned hereinafter are as follows:

#### 1) Enzyme activity

SNDH III of the present invention catalyzes the oxidation of L-sorbose to  
30 vitamin C and/or 2-KGA in the presence of an electron acceptor according to the following reaction equation:



The SNDH III does not work with oxygen as an electron acceptor. This was affirmed by the failure of SNDH III to convert L-sorbose to vitamin C and/or 2-KGA using oxygen as a possible electron acceptor. Furthermore, no oxygen consumption was detected in the reaction mixture as detected with a dissolved oxygen probe. In addition  
5 NAD and NADP are not suitable electron acceptors. However, other conventional electron acceptors can be utilized in conjunction with SNDH III of this invention. Preferred electron acceptors are 2,6-dichlorophenolindophenol (DCIP), phenazine methosulfate (PMS), ferricyanide and cytochrome *c*. There is no minimum amount of electron acceptors which must be present for at least some of the aldehyde substrate to be  
10 converted to its corresponding acid. However, the amount of substrate which can be oxidized depends on the amount of the particular electron acceptor and its electron accepting characteristics.

The enzyme assay was performed as follows:

- a) Assay determining the enzyme activity for the conversion from L-sorbose to each  
15 product, vitamin C or 2-KGA

The reaction mixture consisted of 1.0 mM PMS, 25 mM potassium phosphate buffer (pH 7.0), 1.0  $\mu$ M PQQ, 1.0 mM  $\text{CaCl}_2$ , 50 mM L-sorbose and enzyme solution in a final volume of 100  $\mu$ l water, said reaction mixture was prepared just before the assay. The reaction was carried out at 30°C for 60 minutes unless otherwise stated. The  
20 amount of vitamin C, as the indication for enzyme activity, was measured at a wavelength of 264 nm by a high performance liquid chromatography system (HPLC) which was composed of a UV detector (TOSOH UV8000; TOSOH Co., Kyobashi 3-2-4, Chuo-ku, Tokyo, Japan), a dualpump (TOSOH CCPE; TOSOH Co.), an integrator (Shimadzu C-R6A; Shimadzu Co., Kuwahara-cho 1, Nishinokyo, Chukyo-ku, Kyoto,  
25 Japan) and a column (YMC-Pack polyamine II; YMC, Inc., 3233 Burnt Mill Drive Wilimington, NC 28403, USA). The amount of produced 2-KGA, as another indication for enzyme activity, was measured by HPLC as described above. One unit of the enzyme activity for each production was defined as the amount of the enzyme which produces 1 mg of vitamin C and 2-KGA, respectively, in the reaction mixture.

- 30 b) The photometrical assay of SNDH III

The reaction mixture consisted of 0.1 mM DCIP, 1.0 mM PMS, 50 mM potassium phosphate buffer (pH 7.0), 1.0  $\mu$ M PQQ, 2-100 mM substrate (L-sorbose, D-glucosone, D-glucose, etc.) and enzyme solution in a final volume of 100  $\mu$ l water, said reaction mixture was prepared just before the assay. The reaction was started at 25°C  
35 with L-sorbose, and the enzyme activity was measured as the initial reduction rate of

DCIP at 600 nm. One unit of the enzyme activity was defined as the amount of the enzyme catalyzing the reduction of 1  $\mu\text{mol}$  DCIP per minute. The extinction coefficient of DCIP at pH 7.0 was taken as  $14.2 \text{ mM}^{-1}$ . A reference cuvette contained all the above constituents except for L-sorbose.

- 5 The protein concentration was measured with Protein Assay CBB Solution (Nacalaitesque, Inc. Kyoto, Japan).

## 2) Substrate specificity

- The substrate specificity of the enzyme was determined using the same enzyme assay method as described under 1b) above with the exception of using 100 mM potassium phosphate (pH 7.5) or 100 mM Tris-HCl (pH 9.0) as buffer. The relative activity of SNDH III for D-glucosone (2 mM), D-glucose (100 mM), and D-xylose (100 mM) was higher than that for L-sorbose (2 mM) at both pH 7.5 and 9.0. However, the relative activity for L-sorbose (100 mM), D-sorbitol (100 mM), and L-gulono- $\gamma$ -lactone (100 mM) was lower than 1% of that for L-sorbose at both pH 7.5 and 9.0. These results are presented in Table 1A.

Table 1A

### Substrate specificity of the purified SNDH III

Substrate	Relative activity (%)	
	pH 7.5	pH 9.0
L-Sorbose	100	100
D-Glucosone	1262	391
D-Glucose	4236	522
L-Sorbose	<1	<1
D-Sorbitol	<1	<1
D-Xylose	5545	633
L-Gulono- $\gamma$ -lactone	<1	<1

Table 1B

Deduced products of the oxidation of the substrate indicated in Table 1A

Substrate	Product
L-Sorbose	Vitamin C / 2-KGA
D-Glucosone	D- <i>iso</i> -Ascorbic acid / 2-Keto-D-gluconate
D-Glucose	D-Gluconate
D-Xylose	D-Xylonic acid

## 3) Optimum pH

5        The correlation between the reaction rate of SNDH III and pH values of the reaction mixture was determined by the same assay method as described under 1a) above, with the exception that various pHs and buffers in a concentration of 100 mM were used.

10        SNDH III of the present invention showed relatively high activity for the production of vitamin C at from about pH 6.5 to about 8.0 and high activity for the production of 2-KGA at a pH of about 9.0.

## 4) Effect of temperature

15        The effect of temperature for the enzyme reaction was tested by the same assay method as described under 1a) above, with the exception that various temperatures were used. In both productions of vitamin C and 2-KGA, the enzyme reaction was carried out stable up to at least 50°C.

## 5) Effects of metal ions and inhibitors

20        The effects of metal ions and inhibitors on the L-sorbose dehydrogenase activity of the enzyme were examined by measuring the activity using the same assay method as described under 1b) above. Each compound solution was stirred into the basal reaction mixture and the reaction was started with the addition of SNDH III of the present invention. The results are shown in Table 2.

Table 2

Effect of inhibitors and metals on the activity of the purified SNDH III

Compound	Relative activity (%)
None	100.0
EDTA	83.9
NaN <sub>3</sub>	74.3
Monoiodoacetate	11.9
CaCl <sub>2</sub> ·2H <sub>2</sub> O	101.5
CoCl <sub>2</sub> ·6H <sub>2</sub> O	67.9
CuSO <sub>4</sub>	<1
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·xH <sub>2</sub> O	61.0
NiSO <sub>4</sub> ·6H <sub>2</sub> O	68.5
TiCl <sub>4</sub>	87.9
ZnCl <sub>2</sub>	63.3
MgCl <sub>2</sub>	74.1

Each compound was added to the reaction mixture at a concentration of 1.0 mM, with the exception that the concentrations of EDTA, NaN<sub>3</sub> and monoiodoacetate were 5.0 mM.

As shown in Table 3, Co<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>3+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, and Mg<sup>2+</sup> inhibited the enzyme activity. The addition of 5 mM monoiodoacetate strongly inhibited the enzyme activity. The addition of 5 mM sodium azide weakly inhibited the enzyme activity.

#### 6) Molecular weight

The molecular weight of the SNDH III was measured with a size exclusion gel column (TSK-gel G3000 SWXL; TOSOH Co., Akasaka 1-7-7, Minato-ku, Tokyo, Japan). The enzyme showed two peaks corresponding to the apparent molecular weight of about 190,000 ± 15,000 Da and about 250,000 ± 20,000 Da on the chromatography. On analyzing the purified SNDH III treated with 2% SDS by a 10% SDS-polyacrylamide gel electrophoresis with following CBB-staining, it was shown that SNDH III consisted of two kinds of heterologous subunits having the molecular weight of 75,000 ± 3,000 (α subunit) and 55,000 ± 2,000 Da (β subunit), respectively. This indicates that SNDH III comprises two kinds of subunit structures of the two α and one β subunits, or the two α and two β subunits, in which the α subunit has a molecular weight of 75,000 ± 3,000 Da and the β subunit has a molecular weight of 55,000 ± 2,000 Da.



Both the trimeric and tetrameric forms of SNDH III are active.

#### 7) Prosthetic group

The purified SNDH III (0.1 mg) in 50  $\mu$ l of 100 mM  $\text{NaH}_2\text{PO}_4$ -HCl (pH about 1.0) was added by an equal volume of methanol and mixed well. The sample was centrifuged to remove a precipitate. The resulting supernatant was used for analysis of the prosthetic group. The absorption spectrum of the extract was almost identical with an authentic sample of PQQ (Mitsubishi Gas Chemical, Japan). Its absorbance peaks were found at 251 and 348 nm. Furthermore, by HPLC analysis using a reverse phase column (YMC-Pack Pro C18 AS-312; YMC Co., Ltd) at a wavelength of 313 nm, the extract of SNDH III with methanol showed the same retention time as that of the authentic PQQ.

The detection of heme *c* of the purified SNDH III was attempted by the reduced-minus-oxidized difference spectrum taken by an UV-VIS recording spectrophotometer (Shimadzu UV-2200; Shimadzu Co.). SNDH III was suspended in 50 mM potassium phosphate buffer (pH 7.0) at a concentration of 50  $\mu$ g/ml and SNDH III of dithionite-reduced form and ammonium persulfate-oxidized form were prepared to measure the difference spectrum. The spectrum gave the difference maxima at 552 and 523 nm.

These results strongly suggest that SNDH III has PQQ and heme *c* as prosthetic groups.

#### 8) Effect of substrate concentration

The velocity of the oxidizing reaction with various concentrations of L-sorbose from 1 mM to 8 mM was measured to determine the *K<sub>m</sub>* value for L-sorbose. The Michaelis constants were calculated to be 6.5 mM and 16.8 mM at pHs of 7.5 and 9.0, respectively, from the Lineweaver-Burk plot based on the reaction velocity when DCIP was used as the electron acceptor for the reaction.

#### 9) Purification procedure

The purification of SNDH III is effected by any combination of known purification methods, such as ion exchange column chromatography, hydrophobic column chromatography, salting out and dialysis.

SNDH III provided by the present invention can be prepared by cultivating an appropriate microorganism in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism.

The microorganisms used for the process of the present invention are microorganisms belonging to the genus *Gluconobacter* which are capable of producing aldehyde dehydrogenase as defined herein before.

A preferred strain is *Gluconobacter oxydans*. The strain most preferably used in the present invention is *Gluconobacter oxydans* DSM 4025, which was deposited at the Deutsche Sammlung von Mikroorganismen in Göttingen (Germany), based on the stipulations of the Budapest Treaty, under DSM No. 4025 on March 17, 1987. The depositor was The Oriental Scientific Instruments Import and Export Corporation for Institute of Microbiology, Academia Sinica, 52 San-Li-He Rd., Beijing, Peoples Republic of China. The effective depositor was said Institute, of which the full address is The Institute of Microbiology, Academy of Sciences of China, Haidian, Zhongguancun, Beijing 100080, People's Republic of China.

Moreover, a subculture of the strain has also been deposited at the National Institute of Advanced Industrial Science and Technology (AIST), Japan, also based on the stipulations of the Budapest Treaty, under the deposit No. *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) on March 30, 1992. The depositor is Nippon Roche K.K., 6-1, Shiba 2-chome, Minato-ku, Tokyo, Japan. This subculture is also most preferably used in the present invention.

Thus, it is an object of the present invention to provide an aldehyde dehydrogenase as defined herein before which is derived from *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a subculture or mutant thereof.

Mutants of *G. oxydans* DSM 4025 (FERM BP-3812) or a microorganism belonging to the genus *Gluconobacter* and having identifying characteristics of *G. oxydans* DSM 4025 (FERM BP-3812) may be obtained by treating the cells by means of, for instance, ultraviolet or X-ray irradiation, or a chemical mutagen such as nitrogen mustard or *N*-methyl-*n*'-nitro-*N*-nitrosoguanidine.

Any type of microorganism may be used, for instance, resting cells, acetone treated cells, lyophilized cells, immobilized cells and the like to act directly on the substrate. Any means per se known as a method in connection with the incubation technique for microorganisms may be adopted through the use of aeration and agitated submerged fermentors is particularly preferred. The preferred cell concentration range for carrying out the reaction is from about 0.01 g of wet cell weight per ml to 0.7 g of wet cell per ml, preferably from 0.03 g of wet cell per ml to 0.5 g of wet cell per ml.

The microorganism "*Gluconobacter oxydans*" also includes synonyms or basonyms of such species having the same physico-chemical properties, as defined by the International Code of Nomenclature of Prokaryotes.

The characteristics of *G. oxydans* DSM No. 4025 (FERM BP-3812) are as follows:

- 5 a) production of 2-KGA from sorbose,
- b) ethanol is oxidized to acetic acid,
- c) D-glucose is oxidized to D-gluconic acid and 2-keto-D-gluconic acid,
- d) ketogenesis of polyalcohols,
- e) pellicle and ring growth in mannitol broth (24 hours cultivation) at pH 4 and 5, and
- 10 pellicle growth in glucose broth at pH 4.5,
- f) glycerol is not substantially oxidized to dihydroxyacetone,
- g) production of 2-keto-D-glucaric acid from sorbitol and glucaric acid but not from glucose, fructose, gluconic acid, mannitol or 2-keto-D-gluconic acid,
- h) polymorphic, apparently no flagella,
- 15 i) brown pigment is produced from fructose,
- j) good growth when co-cultured in the presence of *Bacillus megaterium* or a cell extract thereof,
- k) streptomycin sensitive.

The microorganism may be cultured in an aqueous medium supplemented with  
20 appropriate nutrients under aerobic conditions. The cultivation may be conducted at a pH of from about 4.0 to about 9.0, preferably from about 6.0 to about 8.0. The cultivation period varies depending on the pH, temperature and nutrient medium to be used, and is preferably about 1 to 5 days. The preferred temperature range for carrying out the cultivation is from about 13°C to about 36°C, preferably from about 18°C to  
25 about 33°C. A temperature of up to about 50°C might be also suitable for the cultivation of the microorganism.

It is usually required that the culture medium contains such nutrients as assimilable carbon sources, for example, glycerol, D-mannitol, D-sorbitol, erythritol, ribitol, xylitol, arabitol, inositol, dulcitol, D-ribose, D-fructose, D-glucose, and sucrose, preferably D-  
30 sorbitol, D-mannitol and glycerol; and digestible nitrogen sources such as organic substances, for example, peptone, yeast extract, baker's yeast, urea, amino acids, and corn steep liquor. Various inorganic substances may also be used as nitrogen sources, for example nitrates and ammonium salts. Furthermore, the culture medium usually contains inorganic salts, for example magnesium sulfate, potassium phosphate and  
35 calcium carbonate.

An embodiment for the isolation and purification of SNDH III from the microorganism after the cultivation is briefly described hereinafter:

- (1) Cells are harvested from the liquid culture broth by centrifugation or filtration.
- (2) The harvested cells are washed with water, physiological saline or a buffer solution having an appropriate pH.
- (3) The washed cells are suspended in the buffer solution and disrupted by means of a homogenizer, sonicator or French press or by treatment with lysozyme and the like to give a solution of disrupted cells.
- (4) SNDH III is isolated and purified from the cell-free extract of disrupted cells, preferably from the soluble fraction of the microorganism.

A cell free extract can be obtained from the disrupted cells by any conventional technique, including but not limited to centrifugation.

SNDH III provided by the present invention is useful as a catalyst for the production of vitamin C and/or 2-KGA from L-sorbose. The reaction can be conducted at pH values of about 4.5 to about 9.0 for both of vitamin C production and 2-KGA production in the presence of an electron acceptor, for example DCIP, PMS and the like in a solvent such as phosphate buffer, Tris-buffer and the like. For vitamin C production, the best results are usually achieved if the pH is set at about 6.5 to about 8.0 and the temperature is set at about 20 to about 40°C. For 2-KGA production, the best results are usually achieved if the pH is set at about 9.0 and the temperature is set at about 20 to about 50°C.

The concentration of L-sorbose in a reaction mixture can vary depending upon other reaction conditions but, in general, is about 0.5 to 50 g/l, most preferably from about 1 to about 30 g/l.

In the reaction, SNDH III may also be used in an immobilized state with an appropriate carrier. Any means of immobilizing enzymes generally known in the art may be used. For instance, the enzyme may be bound directly to a membrane, granules or the like of a resin having one or more functional groups, or it may be bound to the resin through bridging compounds having one or more functional groups, for example glutaraldehyde.

In addition to the above, the cultured cells are also useful for the production of carboxylic acids and/or its lactones from their corresponding aldoses, especially for the production of 2-KGA and/or vitamin C from L-sorbose. The production of other carboxylic acids and/or its lactones from their corresponding aldoses is carried out under

the same conditions, including substrate concentration, as the conversion of L-sorbose to 2-KGA and/or vitamin C as described above.

The following Examples further illustrate the present invention.

### 5 Example 1: Preparation of SNDH III

All the operations were performed at 8°C, and the buffer was 0.05 M potassium phosphate (pH 7.0) unless otherwise stated.

#### (1) Cultivation of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812)

*Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) was grown on an agar  
10 plate containing 5.0% D-mannitol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.75% corn steep liquor, 5.0% baker's yeast, 0.5% urea, 0.5%  $\text{CaCO}_3$  and 2.0% agar at 27°C for 4 days. One loopful of the cells was inoculated into 50 ml of a seed culture medium containing 2% L-sorbose, 0.2% yeast extract, 0.05% glycerol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.75% corn steep liquor, 0.5% urea and 1.5%  $\text{CaCO}_3$  in a 500 ml Erlenmeyer flask, and cultivated at 30°C with 180 rpm  
15 for one day on a rotary shaker. The seed culture thus prepared was used for inoculating 15 liters of medium, which contained 8.0% L-sorbose, 0.05% glycerol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3.0% corn steep liquor, 0.4% yeast extract and 0.15% antifoam, in a 30-l jar fermentor. The fermentation parameters were 800 rpm for the agitation speed and 0.5 vvm (volume of air / volume of medium / minute) for aeration at a temperature of  
20 30°C. The pH was maintained at 7.0 with sodium hydroxide during the fermentation. After 48 hours of cultivation, 30 liters of the cultivated broth containing the cells of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) by using the two sets of fermentors were harvested by continuous centrifugation. The pellets containing the cells were recovered and suspended in an appropriate volume of saline. After the suspension  
25 was centrifuged at 2,500 rpm (1,000 x g), the supernatant containing the slightly reddish cells was recovered to remove the insoluble materials derived from corn steep liquor and yeast extract which were ingredients for the medium. The supernatant was then centrifuged at 8,000 rpm (10,000 x g) to obtain the cell pellet. As a result, 123 g of the wet weight of cells of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) was obtained  
30 from 30 liters of the broth.

#### (2) Preparation of cytosol fraction

A portion (64.2 g) of the cell paste was suspended with 280 ml of the buffer and passed through a French pressure cell press. After centrifugation to remove intact cells,

the supernatant was designated as the cell-free extract, and the cell-free extract was centrifuged at 100,000 x g for 60 minutes. The resultant supernatant (227 ml) was designated as the soluble fraction of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812). After this fraction was dialyzed against the buffer, 105 ml of the dialyzed fraction  
5 having the specific activity for producing vitamin C from L-sorbose of 0.107 unit/mg protein were used for the next purification step.

(3) Diethylaminoethyl (DEAE)-cellulose column chromatography

The dialysate (150 ml) was put on a column of DEAE-cellulose (Whatman DE-52, 3 x 50 cm; Whatman BioSystems Ltd., Springfield Mill, James Whatman Way,  
10 Maidstone, Kent, U.K.) equilibrated with the buffer and washed with the buffer to elute minor proteins. Then proteins bound to the resin were eluted stepwise with 0.28, 0.32, 0.36 M NaCl in the buffer. Major enzyme activity was eluted at 0.36 M NaCl. The active fractions (143 ml) were collected.

(4) Carboxymethyl-cellulose column chromatography

15 A portion (127 ml) of the active fraction from the previous step was filtrated by an ultrafiltrator (Centriprep-10, Amicon; Amicon Inc. Cherry Hill Drive, Beverly, MA 01915, U.S.A.) to concentrate. After the concentrated sample (28 ml) was dialyzed against the buffer, 28 ml of the dialyzed fraction (31 ml) was put on a column of Carboxymethyl-cellulose (Whatman CM-52, 3 x 23 cm; Whatman BioSystems Ltd.)  
20 equilibrated with the buffer. The proteins that passed through the column without binding to the resin were collected.

(5) Q-sepharose column chromatography (1<sup>st</sup> step)

The pooled active fractions (43 ml) were filtrated by an ultrafiltrator (Centriprep-10) to concentrate. A portion (9.5 ml) of the concentrated active fraction (10 ml) from  
25 the previous step was put on a column of Q-sepharose (Pharmacia, 1.5 by 50 cm) equilibrated with the buffer. After the column was washed with the buffer containing 0.3 M NaCl, a linear gradient of NaCl from 0.3 to 0.6 M was added to the buffer. The active fractions were eluted at NaCl concentrations ranging from 0.55 to 0.57 M.

(6) Q-sepharose column chromatography (2<sup>nd</sup> step)

30 The pooled active fractions (22 ml) from the previous step were filtrated by an ultrafiltrator (Centriprep-10) to concentrate. And the concentrated sample (3.0 ml) was dialyzed against the buffer. The dialyzed sample (3.5 ml) was put on a column of Q-sepharose (Pharmacia, 1.5 by 50 cm) equilibrated with the buffer. After the column was

washed with the buffer containing 0.35 M NaCl, a linear gradient of NaCl from 0.35 to 0.7 M was added to the buffer. The active fractions were eluted at NaCl concentrations ranging from 0.51 to 0.53 M.

(7) Gel filtration column chromatography

5       The pooled active fractions (20 ml) from the previous step were filtrated by an ultrafiltrator (Centriprep-10) to concentrate and desalt. A portion (1.5 ml) of the concentrated and desalted (below 0.1 M NaCl) sample (2.0 ml) was put on a column of Sephacryl S-300 High Resolution (Pharmacia, 1.5 by 120 cm) equilibrated with the buffer containing 0.1M NaCl. The active fractions (12 ml) were collected and dialyzed against  
10   the buffer.

(8) Hydrophobic column chromatography

15       The dialyzed active fraction from the previous step was filtrated by an ultrafiltrator (Centriprep-10) to concentrate. A portion (1.5 ml) of the concentrated sample (1.75 ml) was added to the equal volume (1.5 ml) of the buffer containing 3 M ammonium sulfate (the final concentration: 1.5 M). After centrifugation (15,000 x g) of the sample, the supernatant was loaded on a column RESOURCE ISO (Pharmacia, 1.0 ml) equilibrated  
20   with the buffer containing 1.5 M ammonium sulfate. After the column was washed with the buffer containing 1.5 M ammonium sulfate, the proteins were eluted with the buffer containing a linear gradient of ammonium sulfate from 1.5 to 0.75 M. The active fractions corresponding to SNDH III were eluted at ammonium sulfate concentrations ranging from 1.15 to 1.13 M. The active fractions were dialyzed against the buffer using dialysis cups (Dialysis-cup MWCO 8000, Daiichi pure chemicals, Nihonbashi 3-13-5, Chuo-ku, Tokyo, Japan). Afterward, the fractions were gathered and stored at -20°C.

A summary of the purification steps of the enzyme was given in Table 3.

Table 3

Purification of SNDH III from *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812)

Step	Total activity (units)	Total protein (mg)	Specific activity (units*/mg protein)
Soluble fraction	343.0	3205.2	0.107
DEAE-Cellulose DE52	26.10	120.67	0.216
CM-Cellulose CM52	28.86	105.70	0.273
Q-Sepharose (1 <sup>st</sup> step)	38.94	12.56	3.100
Q-Sepharose (2 <sup>nd</sup> step)	10.77	3.47	3.102
Sephacryl S-300HR	9.09	0.71	12.81
RESOURCE-ISO	4.07	0.12	34.53

One unit\* of the enzyme was defined as the amount of enzyme which produces 1 mg of vitamin C per hour in the reaction mixture described in 1a) mentioned above.

#### (9) Purity of the isolated enzyme

The purified enzyme (0.12 mg/ml) with a specific activity of 34.5 units per mg protein for vitamin C production and a specific activity of 7.82 units per mg protein for 2-KGA production was used for the following analysis:

The molecular weight of the native SNDH III was estimated by high performance liquid chromatography using a size exclusion gel column (TSK gel G3000 SWXL column, 7.8 x 300 mm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl at 280 nm and a flow rate of 1.5 ml per minute. Cyanocobalamin (1.35 kDa), myoglobin (17 kDa), ovalbumin (44 kDa),  $\gamma$ -globulin (158 kDa) and thyroglobulin (670 kDa) were used as molecular weight standards. The purified SNDH III showed two peaks having the molecular weight of  $190,000 \pm 15,000$  Da and  $250,000 \pm 20,000$  Da, respectively.

According to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), SNDH III consisted of two kinds of subunits, which are one with a molecular weight of  $75,000 \pm 3,000$  Da and another with a molecular weight of  $55,000 \pm 2,000$  Da.

#### (10) Identification of the reaction product

The reaction mixture containing the purified SNDH III (1.21  $\mu$ g), L-sorbose (50 mM), PMS (1 mM),  $\text{CaCl}_2$  (1 mM) and PQQ (1  $\mu$ M) in 100  $\mu$ l of the buffer was



incubated for 1 hour at 30°C. The reaction products were analyzed on thin layer chromatography (Silica gel 60F<sup>254</sup>, MERCK, 64271 Darmstadt, Germany) and HPLC. Two kinds of products, vitamin C and 2-KGA, were obtained from the enzyme reaction. For vitamin C, the sample was assayed by an amino-column (YMC-Pack Polyamine-II, YMC, Inc.) on a HPLC system. For 2-KGA, the sample was assayed by a C-18 column (YMC-Pack Pro C18, YMC, Inc.) on a HPLC system.

#### Example 2: Effect of pH on the production of vitamin C or 2-KGA from L-sorbose by SNDH III

The effect of pH for the enzyme reaction was tested. The reaction mixture containing the purified SNDH III (607 ng), L-sorbose (50 mM), PMS (1 mM), CaCl<sub>2</sub> (1 mM) and PQQ (1 µM) in 100 µl of the buffer (100 mM) was incubated for 1 hour at 30°C. The reaction products were analyzed by HPLC. The result is shown in Table 4.

Table 4

#### Effect of pH on the production of vitamin C or 2-KGA from L-sorbose by SNDH III

Buffer	pH	Vitamin C produced (mg/l)	2-KGA produced (mg/l)
Citrate-NaOH	4.47	3.2	0.0
Citrate-NaOH	5.43	6.2	13.9
Citrate-NaOH	6.42	83.2	15.1
Potassium phosphate	6.64	32.2	6.5
Potassium phosphate	7.05	77.8	11.1
Potassium phosphate	7.44	240.9	19.2
Potassium phosphate	7.84	137.9	8.2
Potassium phosphate	8.20	86.6	14.9
Tris-HCl	7.91	103.8	126.9
Tris-HCl	8.41	29.4	126.0
Tris-HCl	8.91	0.0	187.7

#### Example 3: Effect of temperature on the production of vitamin C or 2-KGA from L-sorbose by SNDH III

The effect of temperature on the enzyme activity was tested. The reaction mixture containing the purified SNDH III (607 ng), L-sorbose (50 mM), PMS (1 mM), CaCl<sub>2</sub>

(1 mM) and PQQ (1  $\mu$ M) in 100  $\mu$ l of 25 mM potassium phosphate buffer (pH 7.0) was incubated for 1 hour at various temperatures (20-60°C). The reaction products were analyzed by HPLC. The result is shown in Table 5.

Table 5

5 Effect of temperature on the production of vitamin C or 2-KGA from L-sorbose by  
SNDH III

Temperature (°C)	Vitamin C produced (mg/l)	2-KGA produced (mg/l)
20	229.6	50.9
25	267.9	53.8
30	232.2	49.9
35	295.4	53.4
40	264.6	45.9
50	202.0	54.4
60	24.4	33.2

Claims

1. A purified aldehyde dehydrogenase having the following physico-chemical properties:

5 a) Molecular weight of  $190,000 \pm 15,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and one  $\beta$  subunit) or molecular weight of  $250,000 \pm 20,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and two  $\beta$  subunits), wherein the  $\alpha$  subunit has a molecular weight of  $75,000 \pm 3,000$  Da and the  $\beta$  subunit has a molecular weight of  $55,000 \pm 2,000$  Da;

b) Substrate specificity: active on aldehyde compounds,

10 c) Cofactors: pyrroloquinoline quinone (PQQ) and heme c,

d) Optimum pH: from about 6.5 to about 8.0 (for the production of vitamin C from L-sorbose) or about 9.0 (for the production of 2-keto-L-gulonate from L-sorbose),

15 e) Inhibitors:  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , monoiodoacetate and sodium azide.

2. The aldehyde dehydrogenase according to claim 1, which is derived from a microorganism belonging to the genus *Gluconobacter* which is capable of producing said aldehyde dehydrogenase.

20 3. The aldehyde dehydrogenase according to claim 2, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a subculture or mutant thereof.

4. The aldehyde dehydrogenase according to claim 3, wherein the microorganism is *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a subculture or mutant thereof.

25 5. A process for producing an aldehyde dehydrogenase having the following physico-chemical properties:

a) Molecular weight of  $190,000 \pm 15,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and one  $\beta$  subunit) or molecular weight of  $250,000 \pm 20,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and two  $\beta$  subunits), wherein the  $\alpha$

subunit has a molecular weight of  $75,000 \pm 3,000$  Da and the  $\beta$  subunit has a molecular weight of  $55,000 \pm 2,000$  Da;

- b) Substrate specificity: active on aldehyde compounds,
- c) Cofactors: pyrroloquinoline quinone (PQQ) and heme *c*,
- 5 d) Optimum pH: from about 6.5 to about 8.0 (for the production of vitamin C from L-sorbose) or about 9.0 (for the production of 2-keto-L-gulononic acid from L-sorbose),
- e) Inhibitors:  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , monoiodoacetate and sodium azide,
- 10 which comprises cultivating a microorganism belonging to the genus *Gluconobacter*, which is capable of producing the aldehyde dehydrogenase having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism, and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism.
- 15 6. The process according to claim 5, wherein the reaction is carried out at a pH of from about 4.5 to about 9.0 and at a temperature of from about 20 to about 50°C.
- 7. A process for producing a carboxylic acid and/or its lactone from its corresponding aldose which comprises contacting the aldehyde with the purified aldehyde dehydrogenase having the following physico-chemical properties:
- 20 a) Molecular weight of  $190,000 \pm 15,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and one  $\beta$  subunit) or molecular weight of  $250,000 \pm 20,000$  Da (consisting of a subunit structure of two  $\alpha$  subunits and two  $\beta$  subunits), wherein the  $\alpha$  subunit has a molecular weight of  $75,000 \pm 3,000$  Da and the  $\beta$  subunit has a molecular weight of  $55,000 \pm 2,000$  Da;
- 25 b) Substrate specificity: active on aldehyde compounds,
- c) Cofactors: pyrroloquinoline quinone (PQQ) and heme *c*,
- d) Optimum pH: from about 6.5 to about 8.0 (for the production of vitamin C from L-sorbose) or about 9.0 (for the production of 2-keto-L-gulononic acid from L-sorbose),

e) Inhibitors:  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , monoiodoacetate and sodium azide,

or cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* which is capable of producing the aldehyde dehydrogenase having the above properties in the presence of an electron acceptor.

8. The process according to any one of claims 5 to 7, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a subculture or mutant thereof.

9. The process according to claim 8, wherein the microorganism is *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a subculture or mutant thereof.

10. The process of claim 7, wherein the lactone is vitamin C, the carboxylic acid is 2-keto-L-gulonic acid and the aldose is L-sorbose.

11. The process according to any one of claims 7 to 10, wherein the reaction is carried out at a pH of from about 4.5 to about 9.0 and at a temperature of from about 20 to about 50°C for the production of vitamin C and 2-keto-L-gulonic acid, respectively.

12. The process according to any one of claims 7 to 11, wherein the reaction is carried out at a pH of from about 6.5 to about 8.0 for vitamin C production and of about 9.0 for 2-keto-L-gulonic acid production, and at a temperature of from about 20 to about 50°C for both production ways.

13. The use of the purified aldehyde dehydrogenase of claim 1 in the process for the production of a carboxylic acid and/or its lactone from its corresponding aldose which comprises contacting the aldehyde with said purified aldehyde dehydrogenase or cell-free extract prepared from a microorganism belonging to the genus *Gluconobacter* which is capable of producing said aldehyde dehydrogenase in the presence of an electron acceptor.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/05676

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/02 C12P7/60 C12P17/04 C12R1/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data, PAJ, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 922 759 A (HOFFMANN LA ROCHE) 16 June 1999 (1999-06-16) page 2, line 24 - line 47 page 3, line 5 - line 12 page 6, line 54 page 9; claim 3 --- -/--	1-13

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

10 September 2003

Date of mailing of the international search report

25/09/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Perez, C

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/05676

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAITO Y ET AL: "CLONING OF GENES CODING FOR L-SORBOSE AND L-SORBOSONE DEHYDROGENASES FROM GLUCONOBACTER OXYDANS AND MICROBIAL PRODUCTION OF 2-KETO-L-GULONATE, A PRECURSOR OF L-ASCORBIC ACID, IN A RECOMBINANT G. OXYDANS STRAIN" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 63, no. 2, 1997, pages 454-460, XP000886144 ISSN: 0099-2240 page 454, left-hand column page 456, right-hand column, last paragraph -----	1-13
A	EP 0 832 974 A (HOFFMANN LA ROCHE) 1 April 1998 (1998-04-01) page 2, line 1 -page 3, line 49 -----	1-13

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/05676

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0922759	A	16-06-1999	EP 0922759 A2	16-06-1999
			BR 9805685 A	11-04-2000
			CN 1225390 A	11-08-1999
			JP 11225754 A	24-08-1999
			US 6242233 B1	05-06-2001
			US 2001026933 A1	04-10-2001
<hr/>				
EP 0832974	A	01-04-1998	EP 0832974 A2	01-04-1998
			BR 9704748 A	10-11-1998
			CN 1183472 A	03-06-1998
			JP 10229885 A	02-09-1998
<hr/>				